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Discovery of potential alleles for iron (Fe) toxicity tolerance in rice: Phenotypic and genotypes analysis of doubled haploid lines

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Abstract. *Chrisnawati L, Miftahudin, Utami DW. 2025. Discovery of potential alleles for iron (Fe) toxicity tolerance in rice: Phenotypic and genotypes analysis of doubled haploid lines. Biodiversitas 26: 851-858.* Tidal swamplands present a promising opportunity for expanding rice cultivation. However, iron (Fe) toxicity poses major obstacles to development. To overcome these challenges, it is crucial to develop Fe-tolerant rice genotypes and identify molecular markers, such as single nucleotide polymorphisms (SNPs). This study aimed to identify Fe-tolerant doubled haploid rice genotypes and SNP markers related to Fe toxicity tolerance. The experiment was carried out by cultivating 45 doubled haploid rice lines (BMIP 1-BMIP 33 and BMIP 39-BMIP 50), derived from reciprocal crosses between IR54/Parekaligora and Bio 110/Markuti, under Fe-toxic conditions. Phenotypic evaluations were based on the leaf bronzing symptom (LBS) score and genotype analysis was performed using high-throughput sequencing of 384 SNPs. SNPs correlated with phenotypic data were analyzed using the Tassel 2.0 software, with significance set at a p-value <0.05. The results showed that there were 12 highly Fe-tolerant and 33 lines with moderate tolerance and seven SNPs were identified in proximity to QTLs/genes associated with iron (Fe) toxicity and abiotic stress responses, namely *qFETOX-2*, *OsIRT*, *OsFRO2*, *OsNRAMP5*, and Cyclin-like F-box. Phylogenetic analysis grouped Fe-tolerant lines, including BMIP 25, BMIP 26, BMIP 46, BMIP 47, BMIP 48, BMIP 49, and BMIP 50, with Mahsuri as the positive control, showing shared genetic traits. These results provided valuable markers for breeding programs to obtain Fe-tolerant rice.

Keywords: Doubled haploid rice, Fe tolerant, genomic, leaf bronzing symptom, single nucleotide polymorphisms

Abbreviations: LBS: Leaf Bronzing Symptom, *OsIRT1*: *Oryza sativa* Iron-Regulated Transporter1, *OsFRO2*: *Oryza sativa* Ferric Reductase Oxidase 2, *OsNRAMP5*: *Oryza sativa* Natural Resistance Associated Macrophage Protein 5, SNPs: Single Nucleotide Polymorphisms, QTL: Quantitative Trait Loci

INTRODUCTION

The projected increase in global population and incomes is expected to shift dietary patterns in the next 35 years, showing the need for more food (Fukase and Martin 2020). By 2050, this rising demand for food will require 120% more water and 42% more agricultural land (Bajželj et al. 2014). Several countries, such as Bangladesh, Brazil, Cambodia, China, the European Union, India, Indonesia, Iraq, Pakistan, and Thailand are anticipated to contribute to this expansion in rice cultivation (Rice Outlook 2024).

Tidal swamplands offer significant potential for agricultural development, particularly for rice cultivation in Indonesia (Fahmid et al. 2022; Sari et al. 2023). The country has approximately 33.40 million ha of swamplands, comprising 20 million ha of tidal swamps and 13.40 million ha of non-tidal swamps. Land use is dominated by rice fields, covering 1,181,694 ha (67.51%) of the developed swampland (Azdan et al. 2021). These swamplands provide a strategic opportunity to convert suboptimal land into productive agricultural zones, thereby increasing Indonesia's food security (Sulaiman et al. 2019). To capitalize on the potential, the Indonesian government initiated a program from 2015 to 2019 to expand rice cultivation by opening new rice fields. However, 30% of the newly opened land has been used for actual rice cultivation, suggesting the need for further development efforts to fully realize the agricultural potential (Hatta et al. 2023).

Efforts to expand new rice fields in tidal swamp areas face significant challenges including low soil fertility, particularly in acid sulfate soils (Khairullah et al. 2021). Several studies have reported the poor fertility of newly developed tidal swamplands, which are often susceptible to nutrient imbalances and toxicity issues, particularly iron (Fe). After flooding newly opened rice fields for 3 to 4 weeks, iron oxide concentrations can increase to approximately 600 ppm, while the toxic threshold for rice plants is between 60-300 ppm (Mahender et al. 2019). In acidic soils, Fe is reduced from $Fe^{3+}$to Fe^{2+} (Aung and Masuda 2020), where the elevated concentrations cause toxicity in rice plants. These high concentrations lead to symptoms such as stunted growth and rust-colored spots on older leaves, a condition known as bronzing (Chrisnawati et al. 2016). Leaf bronzing is a major trait for evaluating Fe

toxicity tolerance among the various response characteristics (Aratani et al. 2023). The significant threshold for Fe toxicity in rice plants in tidal swamp areas is approximately 260 ppm, showing the need for developing Fe-tolerant genotypes (Rumanti et al. 2018).

A molecular method for developing Fe-tolerant genotypes and identifying markers essential for rice breeding programs has become increasingly important (Maruapey et al. 2020). High-throughput methods, such as genotypes-by-sequencing, provide the necessary marker coverage by generating a large number of single nucleotide polymorphisms (SNPs) (Ousmael et al. 2023). Genotypes-by-sequencing is a cost-effective method that enables the simultaneous discovery and genotyping of significantly high-quality SNPs at a lower cost, offering a simpler and faster alternative to earlier methods. The association between abiotic stress in rice and SNP genotyping has been widely explored to identify potential markers for rice breeding. For example, recent studies include the identification of heat-tolerance-related genes (Sarker et al. 2024), candidate genes for salinity and anaerobic tolerance at the germination stage (Islam et al. 2022), and significant genomic regions for root and yield-related traits under aerobic and irrigated conditions (Padmashree et al. 2023). Our previous study also utilized SNPs to predict alleles responsible for rice tolerance to Fe; however, it was not accompanied by information on the discovery of Fe-tolerant genotypes (Chrisnawati et al. 2021). This study aimed to identify Fe-tolerant doubled haploid rice genotypes and SNP markers related to Fe toxicity tolerance.

MATERIALS AND METHODS

Plant materials

The plant materials used in this study comprised 45 doubled haploid (DH) rice lines derived from reciprocal crosses between IR54/Parekaligora and Bio 110/Markuti, namely BMIP 1-BMIP 33, and BMIP 39-BMIP 50. A total of 4 rice lines served as the parental lines for the crosses, namely Bio 110, Markuti, IR54, and Parekaligora. Additionally, 2 control plants were included, namely cv. Mahsuri, which served as the control for Fe toxicity tolerance, and cv. IR64 as the control for sensitivity. Mahsuri was selected due to its tolerance to iron (Fe) toxicity and its low leaf bronzing score (LBS) (Nugraha et al. 2016b), while rice cv. IR64 is classified as highly susceptible to iron (Fe) toxicity stress (Turhadi et al. 2019)

Procedures

Planting rice population

The rice population was cultivated in Taman Bogo, East Lampung, Indonesia containing 750 ppm of Fe, in accordance with testing standards for evaluating Fe toxicity tolerance. Subsequently, selection was carried out using the stripe check method. Lines were assessed in a randomized complete block design (RCBD) with two replications, using plots of 1×3 m. Seedlings were transplanted 21-25 days after sowing, with a spacing of 20×20 cm. A total of 3 seedlings per hill were arranged in rows within the experimental plots. Urea fertilizer was applied at a rate of 120 kg/ha in three doses, namely one-third at transplanting, one-third at 4 weeks after transplanting (WAT), and the final third at 7 WAT. Phosphate fertilizer (SP36) was applied at 60 kg/ha at transplanting. However, potassium chloride (KCl) was not used due to the potential to alleviate Fe toxicity by increasing the root's capacity to oxidize excess ferrous ions (Utami and Hanarida 2014).

Fe toxicity evaluation

The phenotypic assessment was performed by screening the rice population with the IRRI bronzing scale (Table 1) to determine their tolerance to Fe toxicity. Leaves showing signs of bronzing were rated according to the IRRI Standard Evaluation System (IRRI 2014) (Figure 1).

DNA extraction

DNA extraction was carried out using the Doyle and Doyle (1987) method with slight modifications, which involves the use of CTAB buffer, chloroform : isoamyl alcohol, and 70% ethanol. The modifications include the addition of sodium acetate (3 M, pH 5.2) and isopropanol. Approximately 0.5 g of rice fresh leaves were homogenized and transferred to a 2 mL eppendorf tube. A total of 2 beads were added and the tube was subjected to liquid nitrogen for 5 minutes to facilitate tissue disruption. The tube was placed in a TissueLyser for 2 minutes at 25 Hz to further homogenize the sample.

Table 1. The leaf bronzing scale is used to determine the level of tolerance to Fe toxicity

Bronzing percent	Score	Description
0	0	Highly tolerant
1-9	1	Tolerant
10-19	2	Tolerant
20-29	3	Tolerant
30-39	4	Moderate
40-49	5	Moderate
50-59	6	Sensitive
60-69	7	Sensitive
70-79	8	Sensitive
80-89	9	Highly sensitive
90-99	10	Highly sensitive

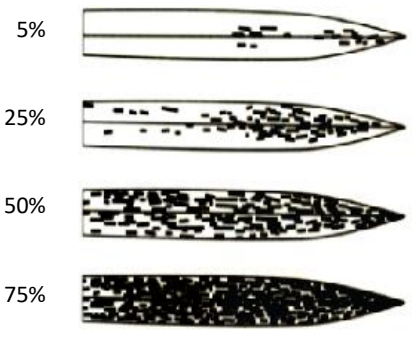

Figure 1. The percentage of leaf damage for assessing the leaf bronzing score (LBS)

After homogenization, 750 µL of CTAB buffer, supplemented with sodium bisulfite (0.38 g in 100 mL CTAB), was added and the mixture was incubated at 60°C for 20 minutes with gentle inversion of the tube every 5 minutes. Subsequently, 750 µL of chloroform : isoamyl alcohol (CI) was added to the sample and centrifuged at 12,000 rpm for 10 minutes using a Beckman Microfuge 12.0. The supernatant (500 µL) was transferred and mixed with 100 µL of 3 M sodium acetate (pH 5.2) and 500 µL of isopropanol. After overnight precipitation in a freezer (-20°C), the sample was centrifuged again at 12,000 rpm for 10 minutes. The supernatant was discarded, followed by washing the pellet with 200 µL of 70% ethanol and centrifuging at 12,000 rpm for an additional 10 minutes. After drying, the pellet was resuspended in 100 µL of TE buffer (Tris-HCl 40 mM, pH 8.3; EDTA 1 mM) and treated with 1.5 µL of RNase. The sample was incubated at 37°C overnight and subjected to RNase inactivation by incubation at 65°C for 15 minutes.

DNA quality was evaluated by electrophoresis on a 0.8% agarose gel using 1x TAE buffer at 100 volts for 60 minutes, with visualization conducted under UV light (Bio-Rad, USA). Subsequently, DNA quantity was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). The DNA concentration was quantified with a NanoDrop spectrophotometer and adjusted to a final concentration of 55 ng/µL for 384 SNPs genotypes on the Illumina iScan GoldenGate platform.

SNPs detection

Genotype profiling was conducted using a custom OPA 384 SNP chip and Illumina's GoldenGate assay kit. The chip was designed based on a set of 384 SNPs, with the selected markers distributed across all 12 rice chromosomes. Genotyping utilized Illumina's Bead Array GoldenGate assay, which relies on 3-micron silica beads arranged in wells etched onto a miniaturized matrix surface, with a spacing of approximately 5.7 microns between beads. Each bead is coated with hundreds of thousands of copies of specific oligonucleotides, which serve as capture sequences for the GoldenGate assay. A high-resolution confocal scanner (iScan) was used to read the arrays and produce intensity data, which were then converted into genotypic data using the built-in GenomeStudio software.

Data analysis

Data on the leaf bronzing symptom (LBS) of rice populations on phenotypic evaluation in the field and SNP genotypes were processed and visualized using Orange Data Mining software. SNPs associated with phenotypic traits were analyzed using a General Linear Model (GLM) approach in Tassel 2.0 software. The significance threshold for the association analysis between genotypes and phenotypic data was set at $p<0.05$. The selected SNPs will be validated through the Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp/index.html) and the Rice Genome Annotation Project (https://rice.uga.edu/). A phylogenetic tree was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) based on the leaf bronzing score and SNP genotypes to identify tolerant lines.

RESULTS AND DISCUSSION

Phenotypic evaluation

Observations suggested that the parental lines had varying levels of tolerance to Fe toxicity, with Markuti and IR54 showing significant resistance. Field trials including 45 DH rice lines also showed a range of responses to Fe stress. Phenotypic assessments identified 12 and 33 DH lines with high and moderate tolerance to Fe toxicity, respectively. The leaf bronzing scores indicate tolerance levels ranging from highly tolerant (Score LBS: 2) to moderate (Score LBS: 5). Green and blue dots indicate Fe tolerance while yellow color indicates moderate Fe tolerance (Figure 2). BMIP 1, BMIP 19, BMIP 20, BMIP 24, BMIP 25, BMIP 26, BMIP 30, BMIP 46, BMIP 47, BMIP 48, BMIP 49, and BMIP 50 showed Fe tolerance, while BMIP 2, BMIP 3, BMIP 14, BMIP 15, BMIP 21, BMIP 27, BMIP 31, BMIP 32, BMIP 33, BMIP 3, BMIP 40, BMIP 41 BMIP 4, BMIP 5, BMIP 6, BMIP 7, BMIP 8, BMIP 9, BMIP 10, BMIP 11, BMIP 12, BMIP 13, BMIP 16, BMIP 17, BMIP 18, BMIP 22, BMIP 23, BMIP 28, BMIP 29, BMIP 42, BMIP 43, BMIP 44, BMIP 4 showed moderate Fe tolerance. Based on the results, Mahsuri showed strong tolerance to Fe toxicity, while IR64 as negative control had high sensitivity.

Genotype analysis

The selected SNPs were identified across all chromosomes, showing genotypes such as AA, TT, CC, and GG. Genotype analysis using 384 SNPs showed heterozygous alleles across multiple loci, with 18% being heterozygous (Table 2). Specifically, these heterozygous alleles can lead to allele segregation, potentially influencing genes associated with Fe stress tolerance. Therefore, there is a need to exclude heterozygous alleles during the selection process for Fe toxicity tolerance to ensure more consistent and reliable identification of tolerant genotypes. Additionally, SNP TBGI069123 was not detected during analysis and was classified as non-called (NC) (Table 2).

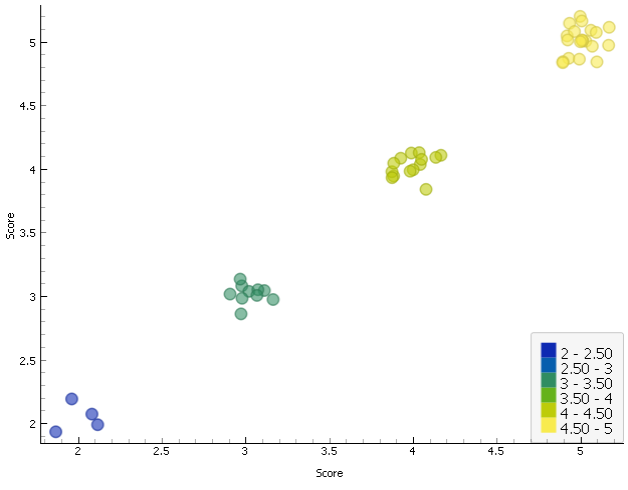

Figure 2. Phenotypic responses observed during field testing of the evaluated lines showed variation in bronzing scores

Association analysis of genotypes and phenotypic

The association analysis between 384 SNP markers and phenotypic responses identified 307 markers with p-values >0.05, while 77 markers had p-values <0.05 (Figure 3). These findings indicate that the 77 SNP markers with p-values <0.05 are associated with tolerance to iron (Fe) toxicity, as determined by leaf bronzing scores. The detailed p-values of the 77 SNP markers are presented in Table 3. Among these 77 markers, only 32 showed polymorphisms between the Fe-tolerant variety (Mahsuri) and the sensitive variety (IR64). These 32 markers were found to be located within genes or QTLs and included in the development of Fe toxicity tolerance. Furthermore, 7 SNPs, distributed across chromosomes 2, 3, 4, 5, 9, and 10, were identified as potential alleles responsible for conferring Fe toxicity tolerance in the BMIP lines (Table 4).

Table 2. Genotype results showed that several BMIP lines had heterozygous alleles (CG, AT, AG, AC) and NC alleles. This suggested variability in the genetic makeup at specific loci associated with Fe toxicity tolerance

SNP	Genotipe	Description	Chromosome
TBGI069137	NC	Non called	1
id2007526	AG	Heterozygote	2
id2011065	AG	Heterozygote	2
TBGI112858	AG	Heterozygote	2
TBGI116973	AG	Heterozygote	2
TBGI129566	AG	Heterozygote	3
TBGI129684	AC	Heterozygote	3
TBGI137370	AG	Heterozygote	3
id5013743	AT	Heterozygote	5
TBGI336735	AT	Heterozygote	7
id8002841	CG	Heterozygote	8
id8001477	AG	Heterozygote	8
id8005361	AG	Heterozygote	8
TBGI367880	AT	Heterozygote	9
id11000784	CG	Heterozygote	11

Table 3. The p-values of 77 SNPs that were less than 0.05, from the association analysis between phenotypic and genotype responses, showed significant correlations

Marker SNP	Locus_pos	Marker_p	Marker SNP	Locus_pos	Marker_p
TBGI278839	329	0.0000004	id11000784	98	0.0063966
TBGI427493	39	0.0000011	id9001029	139	0.006471
TBGI367863	230	0.0000106	TBGI129566	61	0.0069774
TBGI336543	131	0.000015	TBGI367880	187	0.007228
TBGI129684	37	0.0000622	id6002291	228	0.0087425
id6012236	47	0.0000719	id4005120	325	0.009311
id8002632	330	0.0000782	id2002229	4	0.0102809
id1002308	62	0.0001305	id4010396	177	0.0102943
id2011065	301	0.0001784	TBGI034022	83	0.0110312
TBGI272517	285	0.000195	TBGI310247	364	0.011249
id3006808	199	0.0002196	TBGI197086	219	0.0120793
TBGI187416	349	0.0003464	id9006377	383	0.0131667
id4011259	114	0.0004061	TBGI187353	359	0.0133894
TBGI367853	172	0.0004096	id8002841	100	0.0137957
id8001477	362	0.0008306	TBGI446447	26	0.0138431
id8000666	89	0.0008714	id5013743	82	0.0138431
id11008054	247	0.0010784	TBGI067836	314	0.0138431
id7003047	339	0.0016704	TBGI272460	382	0.014174
TBGI367903	252	0.0017523	id6006147	72	0.0151695
id2012408	191	0.0018154	id6015421	306	0.0155536
TBGI446437	106	0.0021281	TBGI187516	60	0.0158087
TBGI336564	358	0.0023924	TBGI117415	165	0.0188501
id12001224	311	0.0024023	id9004788	56	0.0195505
id4010621	6	0.0026414	id8005361	345	0.021287
TBGI116973	48	0.0026414	TBGI336735	121	0.0215788
TBGI427500	263	0.0027511	TBGI204006	336	0.0220803
TBGI187378	160	0.0031264	TBGI446469	198	0.022492
TBGI112898	33	0.0032022	TBGI069137	76	0.0233575
id12001996	29	0.003281	TBGI051917	348	0.0245831
id8001543	44	0.003281	id2007526	175	0.0258661
id10000498	65	0.003281	TBGI335702	235	0.0260672
TBGI137370	229	0.003281	id7000384	182	0.0289093
TBGI112858	291	0.003281	id2000405	282	0.0328071
id7000063	278	0.0034593	TBGI446470	13	0.0340879
TBGI264429	169	0.0037439	TBGI278115	242	0.0369013
TBGI367888	184	0.0051467	TBGI116813	145	0.0406201
TBGI446467	183	0.0052091	id7003748	75	0.0449804
TBGI133961	294	0.0055195	TBGI187513	351	0.0456927
id8001331	144	0.00618			

Table 4. Potential SNPs that identify genes or QTLs associated with the development of Fe tolerance in the BMIP rice population

ID SNP	Chr.	QTL/ Genes
TBGI116973	2	*qFETOX-2*
TBGI137370	3	*OsIRT*
id3006808	3	*OsIRT*
TBGI204006	4	*OsFRO2*
TBGI187378	5	*OsNRAMP5*
id9006377	9	Cyclin-like F-box
id10000498	10	Cyclin-like F-box

Figure 3. The distribution of p-values from the association analysis between phenotypic responses and genotype data is represented with a color gradient. Gray indicates p-values greater than 0.99, yellow and green represent p-values ranging from 0.05 to 0.99, and blue denotes p-values less than 0.05. Out of the 384 SNPs analyzed, 77 showed a significant association, with p-values below 0.05

The phylogenetic tree constructed from 384 SNP genotypes and leaves bronzing scores from phenotypic responses showed 4 major clusters (Figure 4). Mahsuri was found to cluster with Markuti, followed by field-tolerant BMIP lines (cluster 1), which included BMIP 25, BMIP 26, BMIP 46, BMIP 47, BMIP 48, BMIP 49, and BMIP 50. Another group of BMIP lines with Fe toxicity tolerance in the field formed a separate cluster (cluster 3), consisting of BMIP 1, BMIP 19, BMIP 20, BMIP 24, and BMIP 30. However, IR64 clustered with moderately tolerant BMIP lines, including BMIP 23, BMIP 28, BMIP 29, BMIP 42, BMIP 43, and BMIP 45 (cluster 2). Furthermore, the moderate BMIP lines clustered into a single group (cluster 4), which included BMIP5, BMIP6, BMIP7, BMIP8, BMIP11, BMIP12, BMIP13, BMIP14, BMIP15, BMIP17, BMIP32, and BMIP40.

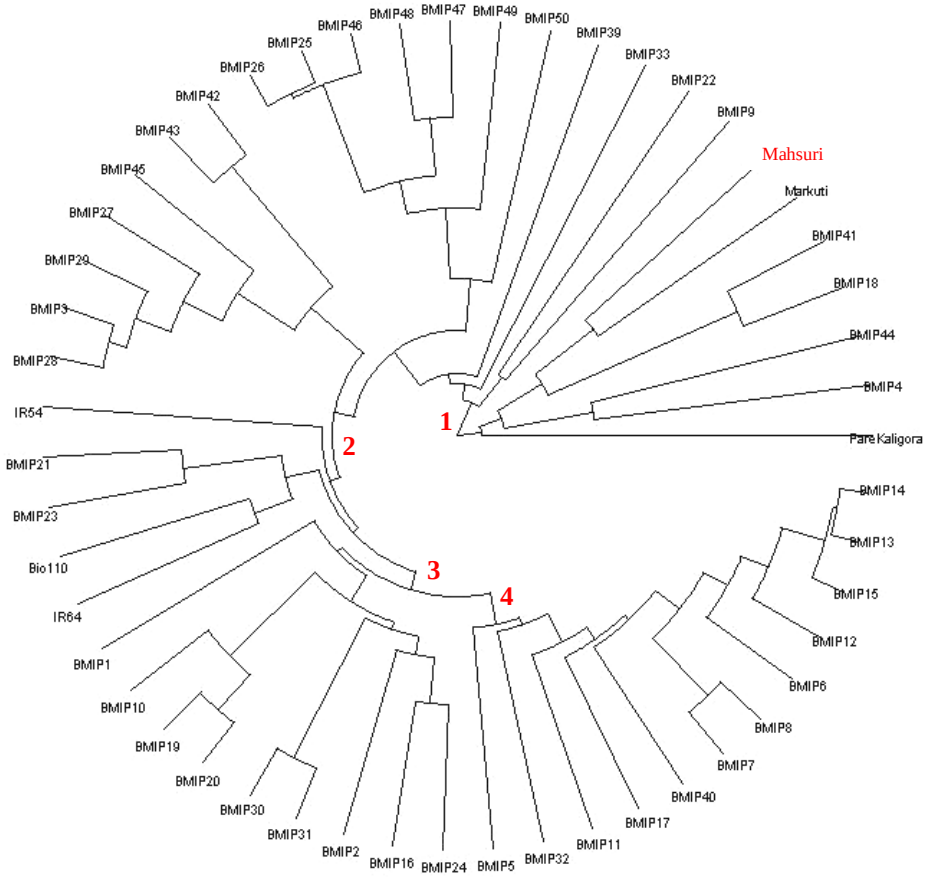

Figure 4. Phylogenetic tree of 45 doubled haploid (BMIP 1-BMIP 33 and BMIP 39-BMIP 50), Bio 110, Markuti, IR54, Parekaligora, Mahsuri, and IR64 resulting from the association of phenotypic scoring

Discussion

Iron toxicity disrupts metabolic processes in rice plants leading to a characteristic brownish-red leaf discoloration, commonly referred to as leaf bronzing score (LBS). The severity of LBS can be a valuable selection criterion in rice breeding programs (Rumanti et al. 2017). Previous studies reported that BMIP parental lines 'Markuti' possessed Fe tolerance due to the presence of Fe tolerance genes (Chrisnawati et al. 2016; Nugraha et al. 2016a). This tolerance trait is inherited in BMIP lines, particularly BMIP 1, BMIP 19, BMIP 20, BMIP 24, BMIP 25, BMIP 26, BMIP 30, BMIP 46, BMIP 47, BMIP 48, BMIP 49, and BMIP 50, which demonstrated tolerance to Fe toxicity in phenotypic field testing (Figure 2). These Fe-tolerant BMIP lines showed lower percentages of leaf damage caused by bronzing in field conditions. Generally, Fe tolerance in rice is a complex trait regulated by multiple genes, showing the importance of conducting genome-wide association studies (GWAS) to explore the relationship between field performance and genotypes (Miao et al. 2024).

Fe toxicity tolerance in BMIP rice populations is attributed to the presence of potential alleles related to Fe tolerance. Therefore, the identification of candidate genes scattered across rice chromosomes has been widely conducted. Anuradha et al. (2012) reported that Fe tolerance-related genes were located on chromosomes 1, 3, 5, 7, and 12. Additionally, quantitative trait loci (QTL) associated with leaf bronzing due to Fe toxicity were mapped across various rice chromosomes. Dufey et al. (2012) identified QTLs on chromosomes 1, 2, 3, 4, 10, 11, and 12 in an F7:8 Recombinant Inbred Lines (RIL) population derived from a cross between Azucena and IR64. Wu et al. (2014) mapped QTLs on chromosomes 1, 3, and 8 in a BC1F5 population from a backcross between Nipponbare and Kasalath.

Candidate genes in this study were identified through SNP analysis, which revealed polymorphisms such as AA, TT, CC, and GG. SNPs with heterozygous alleles (Table 2) were excluded from this study to avoid allele segregation, which could interfere with the identification of genes associated with Fe stress tolerance. The analysis of the association between SNP genotypes and the field resistance of the BMIP lines showed 32 selected SNP markers that exhibited polymorphisms between the Fe-tolerant variety (Mahsuri) and the sensitive variety (IR64). Seven of these SNPs were located in QTL blocks and genes associated with Fe tolerance, as well as other abiotic stress responses (Table 4). On chromosome 2, SNP TBGI116973 was found near the QTL *qFETOX-2*, which originated from an F8 RIL population derived from a cross between IR29 (sensitive) and Pokkali (tolerant) (Wu et al. 2014). The *qFETOX-2* QTL was correlated to bronzing scores, a key indicator of Fe toxicity tolerance (Wu et al. 2014). On chromosome 3, where the *OsIRT* gene was located, 2 significant SNP markers were identified: TBGI137370 and id3006808. The *OsIRT* gene plays a significant role in metal homeostasis, particularly in Fe uptake and partitioning within the plant (Pradhan et al. 2020). The *IRT1* gene helped in Fe^{2+} partitioning, enabling plants to tolerate higher Fe^{2+} levels (Utami and Haranida 2014). According to Chrisnawati et al. (2016), *OsIRT1* and *OsIRT2* were significantly associated with Fe tolerance traits in BMIP rice.

SNP TBGI204006 on chromosome 4 was located near *OsFRO2*, which was associated with Fe tolerance in BMIP rice lines (Chrisnawati et al. 2016). On chromosome 5, SNP TBGI187378 was correlated with the *OsNRAMP* gene, which encoded a metal transporter in Fe uptake and distribution (Li et al. 2021; Chang et al. 2022; Hao et al. 2022; Kanwal et al. 2024). The *OsNRAMP5* gene was considered essential for Fe transport in rice as well as plant growth and development (Ishimaru et al. 2012). Additionally, SNP id9006377 on chromosome 9 and id10000498 on chromosome 10 were associated with genes encoding cyclin-like F-box domain-containing proteins, with SNPs being in a homozygous state. These cyclin-like F-box proteins regulated major physiological processes, including plant growth and response to external stimuli (Xu et al. 2021), and were downregulated in response to excess Fe (Bashir et al. 2014).

The phylogenetic tree based on SNP data and leaf bronzing scores proved useful for selecting Fe-tolerant lines within the BMIP population (Figure 4). The ability of BMIP 25, BMIP 26, BMIP 46, BMIP 47, BMIP 48, BMIP 49, and BMIP 50 to show field tolerance and cluster with Mahsuri suggested their potential as strong candidates. The mechanism of Fe homeostasis regulation in selected BMIP lines included a coordinated network of genes and pathways responsible for Fe uptake, distribution, and detoxification, preventing toxic Fe accumulation. Furthermore, *OsIRT1* gene, located on chromosome 3 was associated with SNPs TBGI137370 and id3006808. *OsIRT1* was observed to play a significant role in regulating Fe^{2+} uptake from the soil and its distribution within plant tissues, preventing Fe accumulation at toxic levels (Song et al. 2024). By controlling Fe uptake, *OsIRT1* maintains adequate concentrations within safe limits. The *OsFRO2* gene, located near SNP TBGI204006 on chromosome 4, also facilitates Fe reduction and transport. *OsFRO* converts Fe from Fe^{3+} to the more readily absorbed Fe^{2+} form, essential for cellular Fe balance and protecting plant cells from oxidative stress caused by high concentrations (Wairich et al. 2024). The presence of *OsNRAMP5* on chromosome 5 associated with SNP TBGI187378, functions as a Fe transporter. *OsNRAMP5* ensures even distribution of Fe across plant tissues, preventing localized Fe toxicity that can damage sensitive areas (Tang et al. 2022). Genes encoding cyclin-like F-box proteins on chromosomes 9 and 10 correlated with SNPs id9006377 and id10000498, contribute to regulating plant growth responses to Fe stress. These F-box proteins modulate essential physiological processes, including growth and adaptation to environmental conditions (Abd-Hamid et al. 2020). Under excessive concentration, the expression of F-box proteins is downregulated, enabling the plant to adjust growth patterns and improve survival in Fe-rich environments. These mechanisms enable selected BMIP lines to maintain Fe homeostasis through uptake regulation, distribution, and physiological adaptation. The complex regulatory network contributes to their tolerance, which is essential for growth in poorly drained.

This study provided valuable information on the genetic basis of Fe toxicity tolerance in rice, a significant challenge in toxic environments, such as Indonesia's tidal swamplands. Through phenotypic evaluations and high-throughput genotypes of 45 DH lines, it was discovered that 12 lines showed high tolerance, while 33 had moderate level. The identification of 77 significant SNP markers, particularly 7 located near QTLs/genes and abiotic stress responses, showed a robust foundation for molecular breeding programs aimed at enhancing Fe tolerance. The phylogenetic analysis showed distinct groupings, where tolerant lines BMIP 25, BMIP 26, BMIP 46, BMIP 47, BMIP 48, BMIP 49, and BMIP 50 clustered with Mahsuri. This showed shared genetic traits related to Fe tolerance, further validating lines as promising candidates for breeding efforts. The results contributed valuable genetic resources for developing rice varieties suitable for Fe-toxic environments, offering a promising strategy for improving food security in regions with suboptimal agricultural land. However, despite these contributions, some limitations should be acknowledged. First, the genetic material used in this study was derived from a limited number of doubled haploid (DH) lines, which typically exhibit lower genetic diversity compared to more heterogeneous populations, such as recombinant inbred lines (RILs) or natural populations. This reduced genetic variation may limit the ability to identify rare alleles or minor genetic variations that could be critical for Fe tolerance. Expanding the population size and incorporating more genetically diverse materials could provide broader insights and improve the robustness of marker validation.

In conclusion, based on the association between field phenotypic data and significant SNP markers linked to Fe tolerance, as well as phylogenetic analysis, Fe-tolerant DH lines were identified, namely BMIP 25, BMIP 26, BMIP 46, BMIP 47, BMIP 48, BMIP 49, and BMIP 50. This study also identified seven significant SNP markers distributed across chromosomes 2, 3, 4, 5, 9, and 10. These SNPs are associated with QTLs/genes, including *qFETOX-2*, *OsIRT*, *OsFRO2*, *OsNRAMP5*, and Cyclin-like F-box. Further studies should focus on validating these markers across larger populations and applying marker-assisted selection (MAS) methods to enhance the breeding of Fe-tolerant rice cultivars, thereby supporting sustainable rice production in Fe-toxic areas.

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